Ceramides Contained in LDL Are Elevated in Type 2 Diabetes and Promote Inflammation and Skeletal Muscle Insulin Resistance

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Dysregulated lipid metabolism and inflammation are linked to the development of insulin resistance in obesity, and the intracellular accumulation of the sphingolipid ceramide has been implicated in these processes. Here, we explored the role of circulating ceramide on the pathogenesis of insulin resistance. Ceramide transported in LDL is elevated in the plasma of obese patients with type 2 diabetes and correlated with insulin resistance but not with the degree of obesity. Treating cultured myotubes with LDL containing ceramide promoted ceramide accrual in cells and was accompanied by reduced insulin-stimulated glucose uptake, Akt phosphorylation, and GLUT4 translocation compared with LDL deficient in ceramide. LDL-ceramide induced a proinflammatory response in cultured macrophages via toll-like receptor–dependent and –independent mechanisms. Finally, infusing LDL-ceramide into lean mice reduced insulin-stimulated glucose uptake, and this was due to impaired insulin action specifically in skeletal muscle. These newly identified roles of LDL-ceramide suggest that strategies aimed at reducing hepatic ceramide production or reducing ceramide packaging into lipoproteins may improve skeletal muscle insulin action. ***Diabetes*** **62:401–410, 2013**

Obesity is associated with the development of chronic diseases, including dyslipidemia, nonalcoholic fatty liver disease, type 2 diabetes, and atherosclerosis. Insulin resistance is a central feature of the pathophysiology of these disorders and is defined as a subnormal response of tissues to the actions of insulin, resulting in decreased glucose uptake into skeletal muscle and impaired suppression of glucose production by the liver. Although the mechanisms responsible for the development of insulin resistance are not fully defined, there is compelling evidence that defective lipid metabolism (1) and consequent subclinical inflammation (2) plays a causative role.

Dyslipidemia resulting from overnutrition and defective adipocyte lipolysis is postulated to be a major contributor to liver and skeletal muscle insulin resistance, at least in part by promoting the intracellular accumulation of lipid metabolites that impair insulin signal transduction (1). Ceramide has been postulated as a primary lipid mediator of skeletal muscle insulin resistance based on findings that intracellular ceramide is elevated in insulin-resistant states (3–5) and that pharmacological inhibition of de novo ceramide synthesis enhances insulin action in insulin-resistant rodents (6). Ceramide induces insulin resistance by inhibiting insulin signal transduction, principally at Akt (7), and possibly via activation of serine/threonine kinases such as Jun NH_2-terminal kinase (JNK) (8), which in turn inhibits activation of insulin receptor substrate proteins (9). Ceramide is also postulated to activate proinflammatory pathways in macrophages, perhaps via amplification of toll-like receptor 4 (TLR4)–mediated inflammation (10,11). Interestingly, activation of TLR4 can increase ceramide levels in macrophages (12), supporting a model whereby ceramide can both induce and amplify macrophage inflammation. Consequently, the activation of tissue-resident macrophages would be predicted to promote a proinflammatory milieu that impairs insulin action.

Although the consequences of tissue ceramide accumulation have been extensively studied over the last decade, it is now apparent that ceramides are also increased in the plasma of obese, type 2 diabetic mice (6,13) and humans (14), and that weight loss induced by gastric bypass surgery (15) or lifestyle modification (16) reduces plasma ceramide. Clinical data indicate that circulating ceramides correlate with systemic insulin resistance and inflammation (14,17), and pharmacological inhibition of whole-body ceramide synthesis in obese mice decreases plasma ceramide, reduces inflammatory parameters, and improves insulin action (6,18). Notably, not all studies report an association between obesity/diabetes and elevated circulating ceramide levels (19–21), and to date, there is no evidence for a direct effect of circulating ceramide on peripheral insulin action and inflammation. In the current study, we show that ceramides contained in LDL are elevated in type 2 diabetes and establish a link between LDL-ceramide, skeletal muscle insulin resistance, inflammation, and impaired systemic insulin action.

RESEARCH DESIGN AND METHODS

Subjects and experimental procedures. Blood samples were collected for plasma ceramide analysis after an overnight fast from lean, insulin-sensitive; obese, insulin-sensitive; and obese, type 2 diabetic individuals. Type 2 diabetes was determined by patients having a fasting plasma glucose >7 mmol/L and/or plasma glucose levels >11.1 mmol/L 2 h after a 75-g oral glucose load. None of the patients received insulin treatment, oral hypoglycemic agents, or statins. We also measured the change in fasting plasma ceramide concentration in 14 obese, nondiabetic women before and after 12% body weight loss via a weight-loss

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program involving dietary caloric restriction and exercise. These subjects participated in a previously published study where details of the weight loss program can be found (22). Protocols were approved by the Alfred Hospital Human Research Ethics Committee and the University of Michigan Institutional Review Board, and were conducted in accordance with the Declaration of Helsinki of the World Medical Association. All volunteers provided written informed consent.

Plasma analysis. Plasma total, LDL, and HDL cholesterol and triglycerides were assayed using a Cholestech L-D-X (Cholestech Corporation, Hayward, CA). Glucose was measured using a Cobas-BIO centrifugal analyzer (Roche) or Thermo DMA kit (Melbourne, Australia). Plasma insulin was measured by radioimmunoassay (Linco, St. Louis, MO) and tumor necrosis factor-α (TNF-α) by ELISA (R&D Systems, Minneapolis, MN).

Fast-protein liquid chromatography isolation of lipoproteins. Plasma lipoproteins were isolated by fast-protein liquid chromatography as described in the Supplementary Methods.

Ceramide determination. Lipids were extracted from lipoprotein fractions (23), the organic phase was evaporated under nitrogen, and ceramides were determined using a radiometric method (γ-[^{32}P]ATP) catalyzed by diacylglycerol kinase. The ^{32}P-labeled ceramides were visualized via autoradiography, and bands were scrapped and quantified by scintillation counting. Plasma ceramide is expressed as μmol/L of ceramide contained in the respective lipoprotein fraction.

Production of LDL-ceramide. Human LDL (Sigma-Aldrich) was mixed with potato starch to form an insoluble complex. The LDL anchored to the potato starch was extracted from endogenous lipids. Ceramide was dissolved in heptane and added to the LDL–potato starch complex. LDL-ceramide was then liberated from the insoluble potato starch by using a polar hydrating solution, and excess potato starch was removed by centrifugation. All steps were performed under nitrogen gas in sterile conditions and are described in the Supplementary Methods. The final ceramide concentration was 1600 nmol/mg LDL protein. Human LDL contains ~6 nmol ceramide/mg LDL protein. The ceramide concentration in culture experiments was 2.1 ± 0.3 μmol/L, which approximates LDL-ceramide in human blood (Fig. 1*D*).

Animal experiments. The Monash University School of Biomedical Sciences Animal Ethics Committee approved all procedures. Male C57BL/6J mice 10 weeks of age were anesthetized with isoflurane, the left jugular vein was cannulated, and the catheter was exteriorized and secured in place. Mice were infused either with 500 μL LDL or LDL-ceramide for 2 h at a rate of 1.33 nmol/min. Mice were housed overnight, and food was removed 4 h before assessment of whole-body and tissue-specific insulin action (24,25). In brief, 2-[1-^{3}H]deoxyglucose ([^{3}H]2DG) (10 μCi) and [U-^{14}C] glucose (2 μCi) were injected intravenously with 0.5 units/kg insulin. Blood glucose and radioactivity were determined over 30 min. Animals were killed by exsanguination, tissues were removed, and tissue-specific glucose uptake was determined from total and phosphorylated [^{3}H]2DG. [U-^{14}C]glucose incorporation into lipid was assessed after lipid extraction, and ^{14}C was counted. Detailed procedures are available in the Supplementary Methods.

Cell culture. L6 GLUT4myc myotubes, RAW 264.7 macrophages, *MyD88*$^{-/-}$/*TRIF*$^{-/-}$ macrophages, and 3T3-L1 adipocytes were grown under standard conditions (Supplementary Methods). Primary hepatocytes were isolated from 6–8-week-old C57BL/6 mice and cultured as previously described (26). Experiments were performed 2 days after isolation. Ceramide secretion experiments were performed in protein-free media as described in the Supplementary Methods.

[^{3}H]2DG uptake. [^{3}H]2DG uptake was determined in LDL or LDL-ceramide–treated cells, without or with 10 nmol/L insulin, as described in the Supplementary Methods.

GLUT4 translocation. GLUT4 translocation was based on an antibody-coupled colorimetric assay as described previously (27); see the Supplementary Methods.

Ceramide uptake experiments. Cells were incubated with LDL or LDL-ceramide for 24 h, washed six times in PBS, lysed in PBS, and sonicated. In

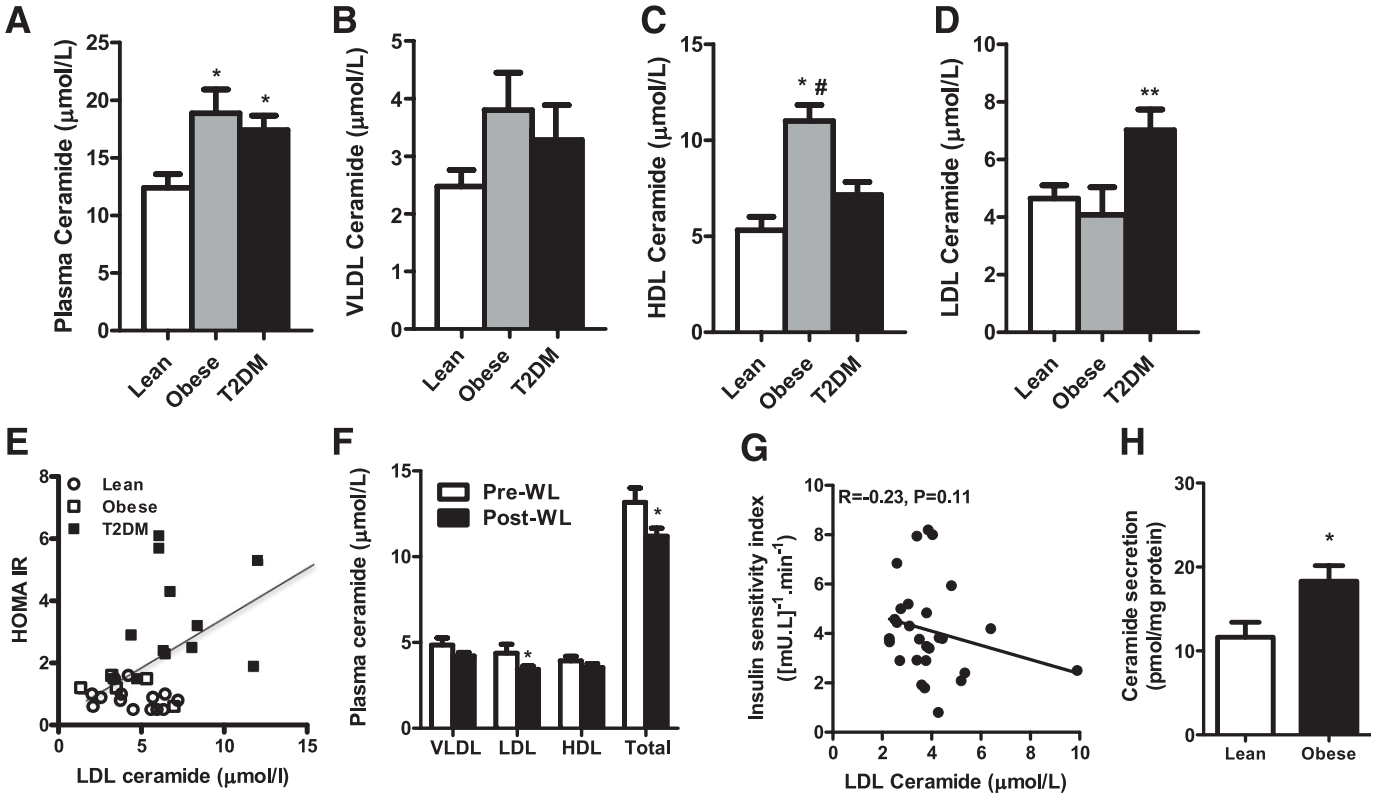

FIG. 1. Plasma ceramides are elevated in type 2 diabetes and reduced with weight loss. Plasma was collected from age-matched lean, insulin-sensitive (*n* = 13); obese, insulin-sensitive (*n* = 5); and obese, type 2 diabetic individuals (T2DM) (*n* = 12). *A*: Plasma ceramide. *B*: Ceramide contained in VLDL. *C*: Ceramide contained in HDL. *D*: Ceramide contained in LDL. *E*: Relationship between LDL-ceramide and HOMA-IR. **P* < 0.05 vs. lean; #*P* < 0.05 vs. T2DM; ***P* < 0.05 vs. lean and obese by one-way ANOVA with Tukey post hoc test. Data are presented as means ± SEM. Plasma was collected from obese, insulin-resistant female subjects before (Pre-WL) and after (Post-WL) losing 12% body mass by diet or diet and exercise. *F*: Plasma ceramide in lipoprotein fractions. Data are presented as means ± SEM. **P* < 0.05 vs. Pre-WL by paired Student *t* test. *n* = 14. *G*: Relationship between LDL-ceramide and insulin sensitivity. *H*: Hepatocytes were isolated from C57BL/6J mice fed a low-fat or high-fat diet. The culture medium was collected between 48 and 72 h, and ceramide secretion was determined by mass spectrometry and normalized to total intracellular protein. *n* = 4 per group. **P* < 0.05 vs. low-fat diet using Student *t* test.

other experiments, cells were treated with 10 μmol/L myriocin or LDL receptor monoclonal (EP1553Y) antibody (Sapphire Bioscience, Waterloo, NSW, Australia) for 1 h prior to and throughout the incubation. Cells were lysed as described above.

Lipid quantification. Mass spectrometry of samples was performed as previously described (28) and in the Supplementary Methods.

Plasma membrane fractionation. Plasma membrane fractions were prepared for immunoblot analysis by differential centrifugation as described in the Supplementary Methods.

Immunoblot analysis. Immunoblot procedures and antibody sources are listed in the Supplementary Methods.

Quantitative RT-PCR. RNA was extracted in Qiazol reagent, isolated using an RNeasy tissue kit (Qiagen), quantified, and reverse transcribed (Invitrogen), and gene products were determined by real-time quantitative RT-PCR (ep realplex Mastercycler; Eppendorf, Hamburg, Germany) using TaqMan Assays-on-Demand (Applied Biosystems). 18S was used as a reference gene. The mRNA levels were determined by a comparative computed tomography method.

Cytokine analysis. Plasma cytokines were assessed using a Milliplex Mouse Cytokine/chemokine 5-PLEX array (Millipore) according to the manufacturer's instructions. Samples were analyzed on a Bioplex 200 system (Bio-Rad). Cytokine secretion was determined by ELISA (R&D Systems).

Statistical analysis. Data are presented as mean ± SEM. One-way ANOVA with Tukey post hoc analysis and Student paired or unpaired *t* tests were used. Correlations were performed using Pearson correlation coefficient. Statistical significance was set at $P < 0.05$.

RESULTS

Circulating ceramides are elevated in patients with type 2 diabetes and negatively correlate with insulin sensitivity. Ceramide is a component of lipoproteins (29) and we show that 98% of plasma ceramides were incorporated into lipoprotein subfractions (Supplementary Fig. 1*A* and *B*). The major species of ceramide (16:0, 22:0, 24:1, and 24:0) show similar profiles across the lipoprotein subfractions (data not shown). The relationship between type 2 diabetes and plasma ceramide is equivocal (14,21). Plasma ceramides were examined in lean, insulin-sensitive; obese, insulin-sensitive; and obese, type 2 diabetic individuals (characteristics in Supplementary Table 1). Total plasma ceramide was higher in obese and type 2 diabetic individuals compared with lean participants (Fig. 1*A*). There was no difference between groups for VLDL-ceramide (Fig. 1*B*), whereas HDL-ceramide was higher in obese, insulin-sensitive participants compared with the other groups (Fig. 1*C*), and LDL-ceramide was 51 and 72% greater in type 2 diabetic individuals compared with lean and obese participants, respectively (Fig. 1*D*). Plasma LDL cholesterol was not different between groups (Supplementary Table 1), and the higher LDL-ceramide did not correlate with plasma LDL cholesterol concentration ($r = 0.03$, $P = 0.90$). apoB-100 was not assessed; hence, we cannot determine whether ceramide in type 2 diabetic individuals is higher per apoB-100.

Total plasma ceramide was associated with insulin resistance (homeostasis model assessment of insulin resistance [HOMA-IR]; $r = 0.35$, $P = 0.05$), and no other significant correlations were evident. Total ceramide did not associate with BMI ($r = 0.24$, $P = 0.25$), suggesting that obesity per se does not account for increased plasma ceramides in type 2 diabetes. A correlation was found for LDL-ceramide versus HOMA-IR ($r = 0.43$, $P = 0.01$) (Fig. 1*E*), and no other significant correlations were detected. Thus, LDL-ceramide is elevated in type 2 diabetes and correlates with insulin resistance, supporting a pathogenic role of LDL-ceramide.

Circulating ceramides were assessed in a separate cohort of abdominally obese women before and after losing 12% of their body mass through lifestyle programs involving weight loss and exercise (Supplementary Table 2). Plasma ceramides were reduced by weight loss, which was attributable to a 22% reduction in LDL-ceramide and nonsignificant reductions in VLDL- and HDL-ceramide (Fig. 1*F*). LDL-ceramide tended to correlate negatively with insulin sensitivity after correction for fat mass (Fig. 1*G*).

Ceramide secretion is elevated in hepatocytes obtained from obese mice. A key question is whether hepatic ceramide secretion is elevated in obesity/insulin resistance. To determine whether caloric overload/obesity increases hepatic ceramide secretion, hepatocytes were isolated from mice fed a low- or high-fat diet for 8 weeks. Ceramide secretion was increased by 57% in hepatocytes obtained from obese compared with lean mice (Fig. 1*H*). The secretion of other lipids, such as sphingomyelin, phospholipids, and dihydroceramide, was not different between groups (data not shown), indicating specificity for ceramide secretion from obese hepatocytes. Notably, the hepatocyte ceramide secretion profile closely matched the plasma ceramide profile in mice. Moreover, ceramide secretion was not detected in cultured myoblasts or 3T3-L1 adipocytes (data not shown). Together, these data indicate that hepatic ceramide secretion is increased in rodent obesity and that the liver is the primary source of circulating ceramide.

Development of a preparation to study LDL-ceramide biology. We created a reconstituted LDL preparation, with or without the addition of ceramide, to investigate the biology of circulating ceramides. This is a relevant approach to assessing circulating ceramide biology and extends on previous studies using short-chain (C2-C6) ceramides, which have major limitations. These include the low abundance of short-chain ceramides in mammalian tissues, their solubility in aqueous solution (i.e., natural ceramide is water insoluble), nonphysiological redistribution within cell compartments, and different signaling compared with long-chain ceramide (30). We incorporated ceramide 24:0 into LDL, because this is the most abundant ceramide species in plasma (29), and ceramide 16:0 in selected studies because this species is considered pathogenic (30). Mass spectrometry analysis confirmed the lipid composition of the LDL preparation (Supplementary Fig. 2*A*). There was no degradation of the apoB-100, the LDL was not oxidized, and there was no evidence of lipopolysaccharide (LPS) contamination or toxicity effects in cells (Supplementary Fig. 2*B*–*F*).

Circulating ceramides cause insulin resistance and inflammation in lean mice. Mice and humans have similar plasma ceramide profiles (Supplementary Fig. 3). To elucidate the physiological role of LDL-ceramide in vivo, LDL-ceramide was infused intravenously for 2 h into lean mice 24 h before assessing insulin-stimulated glucose transport. Ceramide-deficient LDL was infused into control mice. Plasma ceramide was not increased immediately (data not shown) or 24 h after the LDL-ceramide infusion (Supplementary Table 3), owing to the slow infusion rate (4.16 μL/min) and concomitant clearance of LDL-ceramide. The LDL-ceramide infusion reduced whole-body glucose clearance by 29% (Fig. 2*A*), which was accounted for by reduced glucose disposal into skeletal muscle (Fig. 2*B*). This was accompanied by decreased Akt phosphorylation (Fig. 2*C*) but, surprisingly, no change in total muscle ceramide (Fig. 2*D*). Ceramide is hydrophobic and does not diffuse freely in the cell, raising the possibility that the incoming ceramide remained localized to the plasma membrane. Consistent with this notion, ceramide tended ($P = 0.08$) to be elevated in plasma membrane–enriched

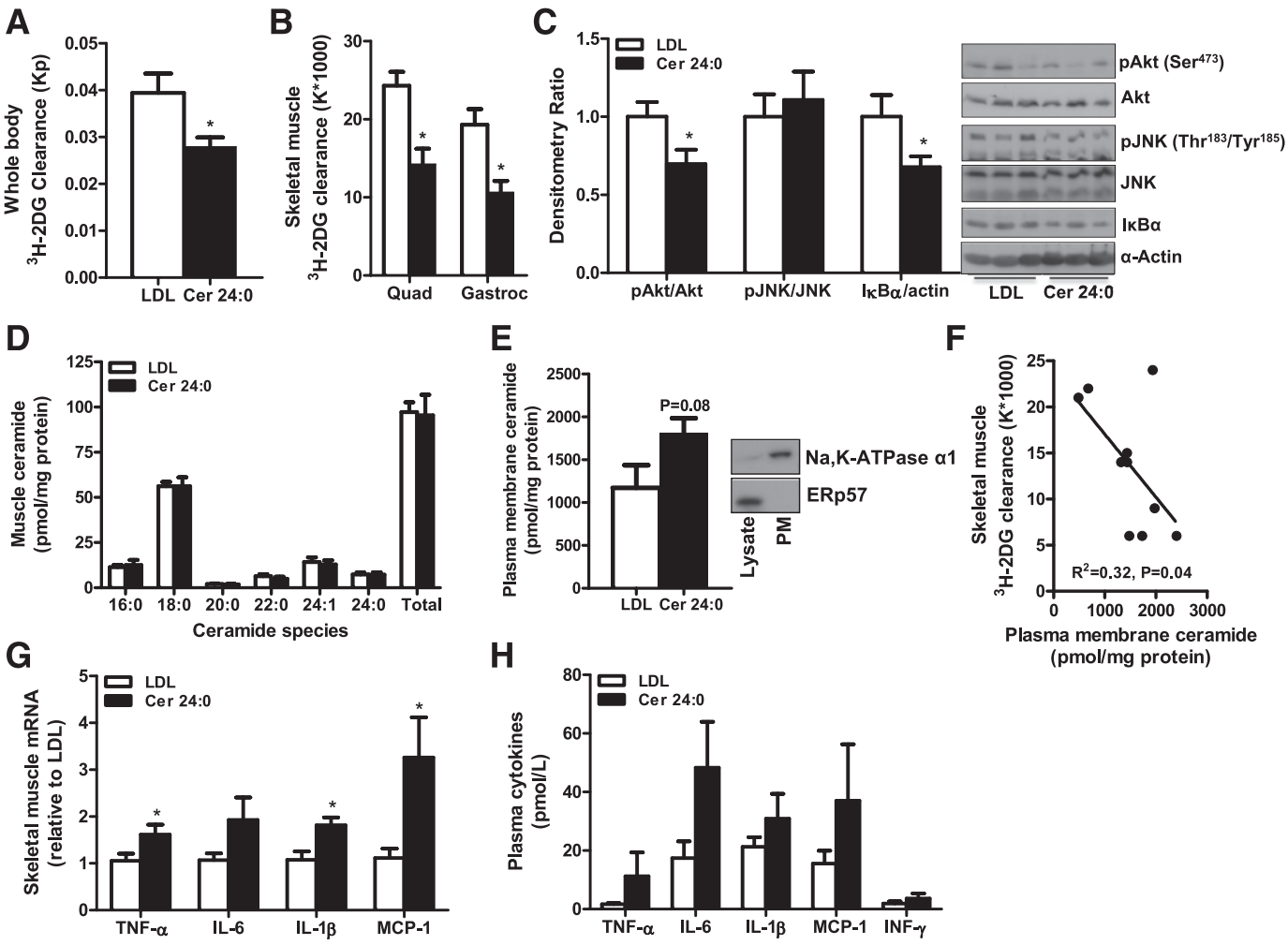

FIG. 2. LDL-ceramide causes insulin resistance in lean mice. C57BL/6J mice 10 weeks of age were infused with LDL or LDL containing C24:0 ceramide (Cer 24:0) for 3 h, and intravenous insulin tolerance tests were performed 24 h later. *A*: Rates of insulin-stimulated [^{3}H]2DG disappearance from the blood. *B*: Rate of insulin-stimulated [^{3}H]2DG clearance into mixed gastrocnemius and mixed quadriceps skeletal muscle. *C*: Quantification of immunoblot analysis of phosphorylated Akt (Ser^{473})/Akt, phosphorylated JNK (The^{183}/Tyr^{185})/JNK and IκBα/α-actin in lysates of skeletal muscle obtained immediately after the intravenous insulin injection. *Right*: Representative immunoblots. pJNK and JNK blots appear as doublets, representing JNK1 and JNK2, respectively. *D*: Ceramide content in mixed quadriceps muscle. *E*: Ceramide content in plasma membrane–enriched fractions of mixed quadriceps muscle. *Right*: Immunoblot of Na,K-ATPase and ERp57 demonstrating enrichment of the plasma membrane fraction and no contamination with endoplasmic reticulum. *F*: Rate of [^{3}H]2DG clearance into skeletal muscle correlated with plasma membrane ceramide concentration. *G*: Transcript levels of proinflammatory cytokines in mixed vastus lateralis skeletal muscle. *H*: Plasma cytokines. Data are presented as means ± SEM (n = 8 for LDL; n = 9 for Cer 24:0). *P < 0.05 vs. LDL by unpaired Student t test.

fractions of skeletal muscle obtained from LDL-ceramide–infused mice (Fig. 2*E*) and was negatively correlated with muscle glucose uptake (Fig. 2*F*).

Intracellular ceramide activates IKKβ/JNK signaling (8,31), and we reasoned that circulating ceramides might also regulate these pathways. LDL-ceramide increased IKKβ signaling in muscle, as assessed by IκBα degradation, but did not affect JNK phosphorylation (Fig. 2*C*). The expression of nuclear factor-κB (NF-κB) target genes was also increased (Fig. 2*G*), and plasma proinflammatory cytokines tended to increase with LDL-ceramide (Fig. 2*H*). The insulin-desensitizing effects of LDL-ceramide were specific to skeletal muscle because glucose disposal into other tissues was not different between groups (Supplementary Fig. 4*A*–*D*). Insulin-stimulated hepatic lipogenesis (glucose→lipid) was also unaffected by LDL-ceramide (Supplementary Fig. 4*E*). Taken together, these experiments indicate that LDL-ceramide induces insulin resistance in lean mice by inhibiting insulin action specifically in skeletal muscle.

LDL-ceramide reduces insulin-stimulated glucose uptake in cultured myotubes. We directly tested the effect of LDL-ceramide on insulin sensitivity in skeletal muscle, the primary site for insulin-mediated glucose disposal in vivo. L6 GLUT4myc myotubes were pretreated for 6 or 24 h with LDL or LDL-ceramide, followed by measurement of 2DG transport. LDL alone mildly increased basal and insulin-stimulated 2DG uptake in myotubes; however, the insulin-stimulated component of 2DG uptake was not different from untreated myotubes (Supplementary Fig. 5*A*). LDL-ceramide did not influence basal-state glucose uptake. Insulin sensitivity was unaffected by 6-h LDL-ceramide treatment (Fig. 3*A*). In contrast, 24 h exposure to LDL-ceramide decreased insulin-stimulated glucose uptake by ~25% (Fig. 3*B* and *C*). Consistent with these results, LDL-ceramide decreased the insulin-stimulated gain in Akt phosphorylation and GLUT4myc, representing decreased GLUT4 translocation and fusion into the plasma membrane (Fig. 3*D* and *E*). LDL-ceramide did not influence extracellular signal–related kinase (ERK), JNK, or

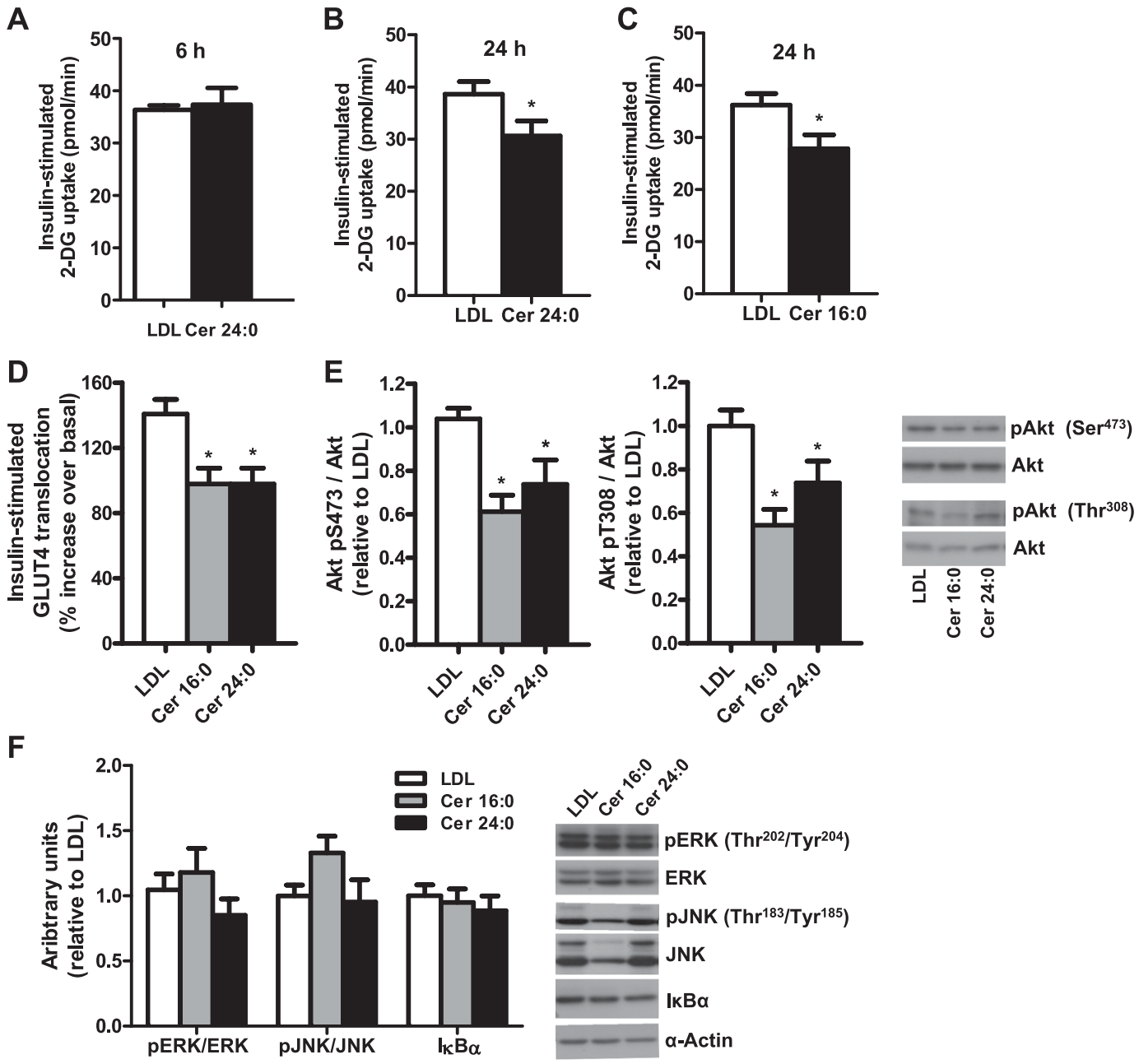

FIG. 3. LDL-ceramide causes skeletal muscle insulin resistance, independent of inflammation. L6 muscle cells stably expressing myc-tagged GLUT4 were incubated with LDL or LDL-ceramide. *A*: LDL-ceramide administration does not influence insulin-stimulated glucose uptake compared with untreated cells after 6 h treatment (n = 6 per group). LDL-ceramide 16:0 and 24:0 (Cer 16:0 and 24:0) treatment for 24 h decreases insulin-stimulated glucose uptake (*B* and *C*) (n = 9 per group), GLUT4 translocation (*D*), and Akt phosphorylation (Ser^{473} and Thr^{308}) (*E*). *Right*: Representative immunoblots. n = 9 per group. *$P < 0.05$ vs. LDL. *F*: LDL-Cer 16:0 and 24:0 has no effect on ERK, JNK, and NF-κB activation as determined by immunoblot analysis (n = 6–12 per group).

NF-κB signaling (Fig. 3*F*). There was no effect of LDL-ceramide on insulin sensitivity in cultured hepatocytes and adipocytes (Supplementary Fig. 5*B* and *C*), which agrees with the in vivo studies (Fig. 2).

LDL-ceramide is taken up by myotubes. Muscle cells were incubated with LDL-ceramide for 24 h to determine whether ceramide contained in LDL can accumulate in myotubes. Exposure to LDL-ceramide 24:0 increased total intramyocellular ceramide content, which was entirely accounted for by increased ceramide 24:0 (Fig. 4*A*). Similarly, intramyocellular ceramide 16:0 was increased after exposure to LDL-ceramide 16:0 (Fig. 4*A*). Muscle sphingomyelin content was not decreased by LDL-ceramide, suggesting that the increase in ceramide was not due to increased sphingomyelinase activity (Fig. 4*B*). The increase in the specific ceramide species was not due to de novo synthesis because there were no fatty acids present in the culture medium, and intramyocellular C24:0 ceramide increased when cells were incubated with LDL-ceramide 24:0, even in the presence of myriocin, a pharmacological inhibitor of serine palmitoyl transferase (Fig. 4*C*). Finally, pretreating myotubes with an LDL receptor monoclonal blocking antibody prior to adding LDL-ceramide 24:0 did not reduce intracellular accumulation of ceramide 24:0 (Fig. 4*D*). Interestingly, intracellular ceramide increased when L6-GLUT4myc cells were treated with the LDL

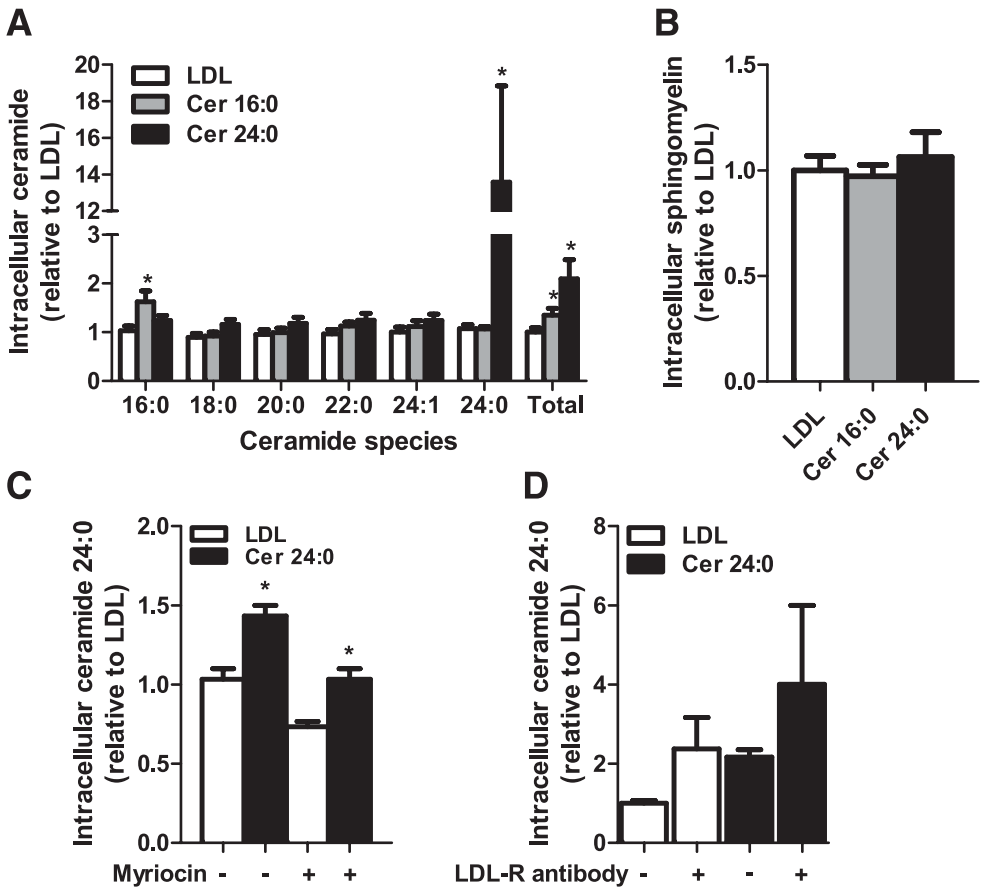

FIG. 4. LDL-ceramide accumulates in skeletal muscle. Intramyocellular ceramide (n = 12 per group) (*A*) and sphingomyelin content (n = 6 per group) (*B*) in myotubes. Each individual experiment was performed in triplicate. *P < 0.05 vs. LDL by unpaired Student t test. *C*: Myotubes were pretreated with the SPT inhibitor myriocin, which blocks de novo ceramide synthesis, or vehicle for 10 min. Thereafter, myotubes were incubated with LDL or LDL-ceramide 24:0 (Cer 24:0) for 24 h, and intracellular ceramide was analyzed. n = 3 per group. *P < 0.05 vs. LDL by two-way ANOVA with Tukey post hoc analysis. *D*: Myotubes were pretreated with a monoclonal rat LDL receptor antibody or denatured antibody for 10 min. Thereafter, myotubes were incubated with LDL or LDL-Cer 24:0 for 24 h, and intracellular ceramide was analyzed. n = 3 per group. Data were analyzed by two-way ANOVA. All data are presented as means ± SEM.

receptor antibody. Collectively, these data show that LDL-ceramide appears to accumulate in myotubes by an LDL receptor–independent event.

LDL-ceramide accumulates in macrophages and causes inflammation. Proinflammatory cytokines are secreted from immune cells residing in adipose tissue and liver (and possibly muscle), and may promote inflammation and insulin resistance in nearby cells (2). Intracellular ceramide production is inextricably linked to inflammation, and short-chain ceramides are putative ligands for the TLR4 (32). Moreover, ceramide contains a fatty acid moiety (C16-C24) that resembles the saturated fatty acids within the lipid A–activating component of LPS, the TLR4 agonist, and ceramide and LPS share other structural similarities (33). The strong tendency for increased plasma cytokines with LDL-ceramide infusion in mice (Fig. 2*H*) and the positive correlation between ceramide and TNF-α in human plasma shown here (Fig. 5*A*), and previously (14,17), led us to ask whether circulating ceramides cause inflammation in macrophages.

We incubated RAW264.7 macrophages with LDL, LDL-ceramide, the potent TLR4 activator LPS, and palmitate, a lipid known to activate TLR4 (34). We first assessed the temporal pattern of activation of proinflammatory protein kinases. LPS rapidly increased JNK phosphorylation, which is a marker of JNK activation (Fig. 5*B*). Although LDL-ceramide increased JNK phosphorylation, this did not occur until 4 h. The time course of these effects suggested that the actions of LDL-ceramide were unlikely to be mediated via ligand activation of the TLRs (which occurs in minutes), rather via the uptake and/or metabolism of the circulating ceramide. In contrast, LDL-ceramide rapidly activated NF-κB signaling (Fig. 5*C*). The expression of several classically activated M1 inflammatory genes, including *TNF-α* and *CCL2*, was increased by LDL-ceramide, and interleukin-6 (IL-6) tended to increase (Fig. 5*D*). The M2 anti-inflammatory gene *IL-10* was decreased by LDL-ceramide (Fig. 5*D*). Further, the addition of LDL-ceramide increased the secretion of TNF-α and IL-6 into the culture medium (Fig. 5*E*). Thus, LDL-ceramide induced a shift toward a proinflammatory profile in macrophages.

Macrophages efficiently transport fatty acids (35) and other atherogenic lipids, and intracellular lipids accumulate in adipose tissue macrophages in obesity (36). LDL-ceramide increased the intracellular ceramide content of macrophages in a species-dependent manner (Fig. 5*F*). The ceramide increase did not coincide with decreased sphingomyelin (data not shown). Thus, ceramide from LDL appears to be taken up by and accumulate in macrophages.

TLR signaling is not required for LDL-ceramide–mediated inflammation in macrophages. A direct interaction between lipid-driven signals (e.g., the fatty acid

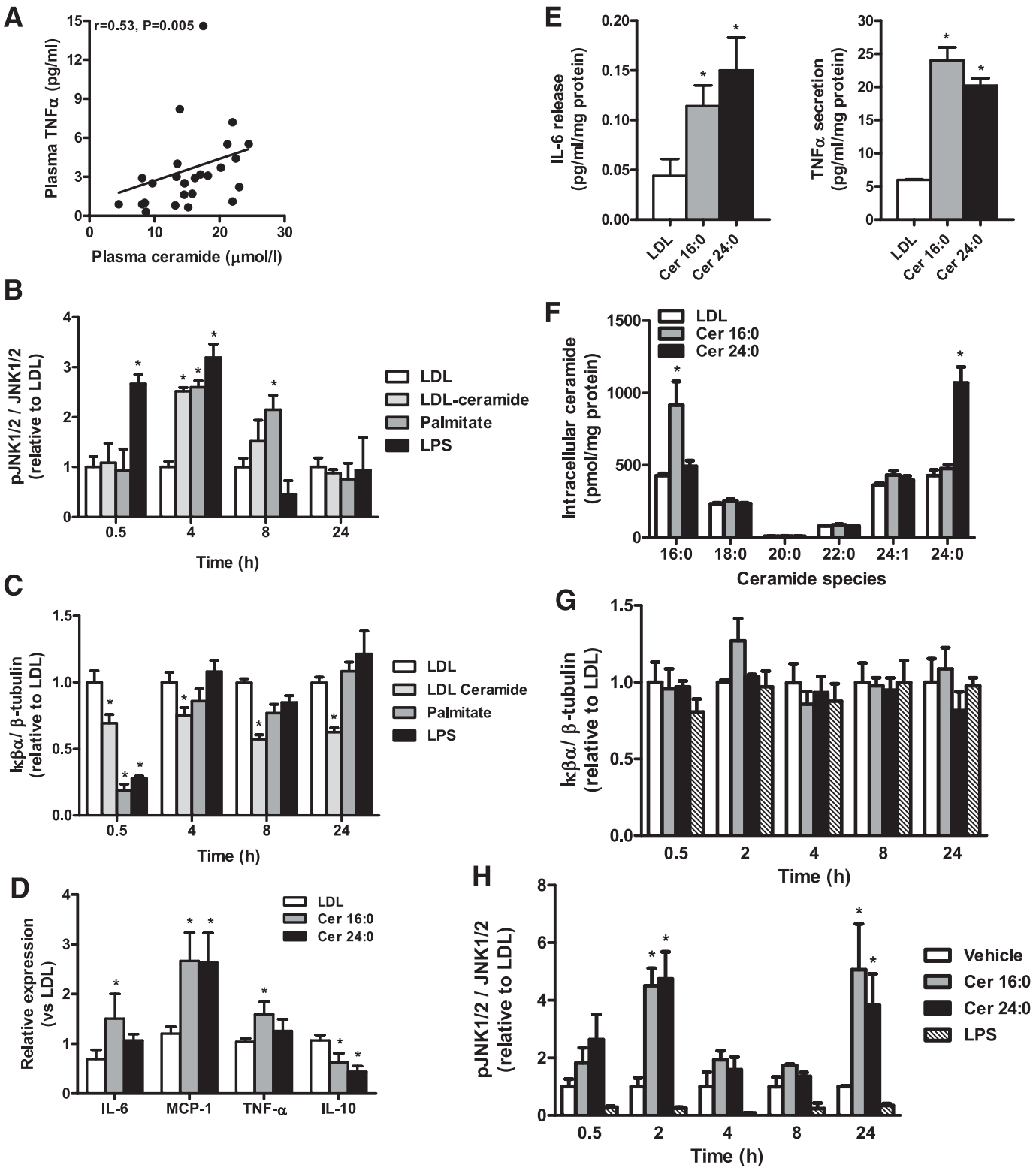

FIG. 5. LDL-ceramides contribute to an M1 phenotype in cultured macrophages. Relationship between plasma ceramide and plasma TNF-α from age-matched lean, insulin-sensitive (n = 10); obese, insulin-sensitive (n = 5); and obese, type 2 diabetic individuals (n = 10). *B* and *C*: RAW 264.7 macrophages were incubated with LDL, 100 nmol/L LDL-ceramide, 100 ng/mL LPS, or 0.75 mmol/L palmitate for the indicated time. Cells were lysed and assessed for JNK phosphorylation (*B*) or IκBα (*C*) by immunoblot analysis. n = 3 per group. *P < 0.05 vs. LDL by one-way ANOVA and Tukey post hoc analysis. Representative blots can be found in Supplementary Fig. 6. *D*: Cells were treated with LDL or LDL-ceramide for 24 h. RNA was extracted and reverse transcribed, and mRNA content was assessed by quantitative RT-PCR. All data were normalized to 18S. n = 9 per group. *P < 0.05 vs. LDL by unpaired Student t test. *E*: Cells were treated with LDL or LDL-ceramide for 24 h and washed, and the culture medium was replaced with no additions. The medium was collected after 24 h, and TNF-α and IL-6 were analyzed by ELISA. n = 3 per group. *P < 0.05 vs. LDL by unpaired Student t test. *F*: Macrophages were treated with LDL or LDL-ceramide for 24 h, cells were lysed, lipids were extracted, and ceramide species were quantified by mass spectrometry. n = 6 per group. *P < 0.05 vs. LDL by unpaired Student t test. *G* and *H*: *MyD88*$^{-/-}$/*Ticam1*$^{-/-}$ macrophages were incubated with LDL, 100 nmol/L LDL-ceramide, or 100 ng/mL LPS for the indicated time. Cells were lysed and assessed for IκBα levels (*G*) or JNK phosphorylation (*H*) by immunoblot analysis. n = 3 per group. *P < 0.05 vs. LDL. All data are presented as means ± SEM by one-way ANOVA and Tukey post hoc analysis. Representative blots can be found in Supplementary Fig. 6.

palmitate) and inflammation occurs via TLR4 activation (34). This interaction may extend to circulating ceramide. Accordingly, we investigated whether TLR signaling mediated the ceramide-induced inflammatory responses by utilizing macrophages deficient in MyD88 (*MyD88*$^{-/-}$) and TRIF (*Ticam1*$^{-/-}$), which prevents propagation of all TLR signaling. IκBα degradation was not affected by LPS or LDL-ceramide in *MyD88*$^{-/-}$/*Ticam1*$^{-/-}$ macrophages, suggesting a dependency of TLR signaling for LDL-ceramide effects on NF-κB signaling (Fig. 5*G*). These experiments do not distinguish which TLR mediates the inflammatory signaling. Conversely, JNK phosphorylation was increased by LDL-ceramide and not LPS (Fig. 5*H*), suggesting that TLR signaling is not required, but rather the uptake/metabolism of ceramide is required, to activate JNK signaling. The increase in JNK was not sufficient to induce changes in TNF-α or IL-6 secretion (Supplementary Fig. 7).

DISCUSSION

This report provides evidence demonstrating a role for LDL-derived plasma ceramides in the development of skeletal muscle insulin resistance and macrophage inflammation, findings that support the hypothesis that ceramide signals generated both from within skeletal muscle and those circulating in LDL contribute to the pathophysiology of whole-body insulin resistance.

The presence of ceramide in the circulation has been known for decades (37), and we detected ceramide in human plasma with concentration and lipoprotein distribution patterns consistent with others (15,29). Several groups have implicated plasma ceramide in the pathogenesis of insulin resistance and systemic inflammation (6,13–15,17,38). Here, we have expanded on these observations and show that ceramide is elevated specifically in the LDL of type 2 diabetic patients compared with insulin-sensitive individuals, independent of obesity. Moreover, weight loss in obese, insulin-resistant individuals reduced LDL-ceramide proportionally more than decreases in VLDL- and HDL-ceramide. The relatively small sample size is a limitation, and our findings require confirmation in larger cohorts.

To examine the relationship between circulating ceramide and insulin resistance, we reconstituted ceramide with LDL and examined insulin action. LDL-ceramide caused whole-body insulin resistance in lean mice, an effect mediated by decreased insulin action in skeletal muscle. The tissue-specific effect was surprising but was supported by in vitro studies demonstrating reduced insulin-stimulated glucose uptake in myotubes, without effects in hepatocytes or adipocytes. Ceramide inhibits insulin signaling by several independent mechanisms: by increasing PP2A activity (39), which decreases Akt phosphorylation and activity (40); by blocking the recruitment of Akt to the plasma membrane (7), which is required for activation by the upstream kinases PDK1 (at Akt Thr^{308}) and TORC2 (at Akt Ser^{473}); and by ceramide accumulating in caveolin-enriched domains, activating PKCζ, which sequesters Akt in a repressed state within these membrane domains to prevent insulin signaling (41). The reduction in insulin-stimulated glucose uptake with LDL-ceramide administration in vivo was accompanied by ceramide accrual in the plasma membrane and reduced Akt phosphorylation. Aside from effects on signaling pathways, ceramide can inhibit Rac activation in the pathway toward GLUT4 translocation (42). Ceramide also forms microdomains in both fluid- and gel-state phosphatidylcholine membranes (43), and altering plasma membrane fluidity may attenuate GLUT4 fusion into the plasma membrane. The decrease in plasma membrane GLUT4 with LDL-ceramide treatment is consistent with these possibilities.

Ceramide initiates signaling pathways that activate JNK and IκKβ (44), proteins that negatively regulate proximal insulin signaling. LDL-ceramide did not affect JNK phosphorylation, although NF-κB signaling and cytokine expression were increased in skeletal muscle in vivo. This proinflammatory effect is unlikely to result from direct LDL-ceramide action in skeletal muscle because we found no evidence of IKK/NF-κB activation in cultured myotubes. Instead, the activation of NF-κB signaling is likely to be mediated by increased plasma cytokines produced by other tissues or, possibly, muscle-resident immune cells. Collectively, our studies show that LDL-ceramides induce insulin resistance, directly, by accumulating in the plasma membrane and interfering with Akt signaling and GLUT4 action and, indirectly, by inducing proinflammatory cytokine production in immune cells. Additional work is required to distinguish the precise mechanism(s) mediating the negative actions of LDL-ceramide on skeletal muscle insulin action.

Although some skepticism persists, there is good evidence linking intracellular ceramide accumulation to insulin resistance, particularly in skeletal muscle (9). The controversy relating to the role of ceramide effects on insulin action may relate to the relatively small changes in muscle ceramide in insulin-resistant states (9) and the likelihood that ceramide is enriched in specific cell regions, which would influence cell signaling with unappreciated specificity (41). For example, *Sptlc2* haploinsufficient mice have increased liver ceramide content, yet plasma membrane ceramides are reduced with concomitant improvements in hepatic insulin signaling (45). In the present studies, total muscle ceramide was unchanged but plasma membrane ceramide was increased with LDL-ceramide administration. LDL can be transported into cells by receptor-mediated and receptor-independent processes; however, skeletal muscle takes up virtually no LDL by receptor-mediated processes (46). In addition, receptor binding is not required for lipid-soluble, LDL-associated products to be transferred to muscle cells (e.g., α-tocopherol) (47), suggestive of lipid-soluble exchange of products between the LDL and the muscle plasma membrane lipids. Although not definitive, our studies showing that ceramide accumulates in myotubes with LDL-ceramide in the presence of an LDL receptor–neutralizing antibody supports this notion. Ceramide has limited transbilayer movement and stays tightly bound to the membrane (48), so if ceramide is produced/obtained from the exoplasmic side (as is the case with LDL-ceramide), then ceramide would more likely act as a structural component of the plasma membrane rather than a signaling molecule. This would likely alter the biophysical properties of the membrane and affect membrane protein topology and function.

Ceramides and inflammation are inextricably linked. On one hand, ceramide is a common molecular intermediate for conveying inflammatory signals. TNF-α increases cellular ceramide via de novo synthesis or sphingomyelin hydrolysis, and IL-1 is a potent inducer of ceramide (9). Activation of the innate immune receptor TLR4 increases SPT transcription and activity, ceramide synthesis, and

plasma lipoprotein ceramide levels (49,50). Many of the cytokines produced by TLR4 also promote ceramide synthesis (e.g., TNF-α), thereby invoking multiple ceramide-producing stimuli. Most inflammatory mediators of ceramide production converge on the IκKβ–NF-κB pathway. On the other hand, ceramides induce inflammatory signals such as the IκKβ–NF-κB pathway. Ceramide causes NF-κB activation (51,52) and TNF-α, IL-6, IL-1β, and MCP-1 are controlled by NF-κB. Blocking ceramide synthesis or inhibiting NF-κB reduces TNF-α and IL-6 production (10,53), although this is not a universal finding (31).

Plasma ceramides are associated with proinflammatory cytokines in individuals with cardiovascular disease (17), type 2 diabetes (14), and obesity (15,38). We confirmed the positive relationship between plasma ceramide and TNF-α in humans and used the LDL-ceramide preparation to determine causality. LDL-ceramide stimulated proinflammatory cytokine production and secretion via several mechanisms: first, by promoting the uptake and accumulation of ceramide that is temporally associated with JNK signaling and, second, by activation of NF-κB signaling that is dependent upon TLR signaling. These in vitro observations agree with the increased cytokine production in mice infused with LDL-ceramide. Thus, moderate increases in LDL-ceramide can induce proinflammatory actions in macrophages and may contribute to inflammation in obesity.

Overall, our data establish that circulating ceramides are elevated in obese, insulin-resistant individuals, and that LDL-ceramide can be reduced with diet and exercise, commonly prescribed lifestyle interventions for patients with type 2 diabetes. The finding that LDL-ceramide can accumulate in the plasma membrane of skeletal muscle, cause insulin resistance, and induce macrophage inflammation substantiates correlative data implicating ceramide in obesity-associated insulin resistance and inflammation, respectively. These newly recognized roles of LDL-ceramide suggest that strategies aimed at limiting hepatic ceramide production or reducing ceramide packaging into lipoproteins may improve skeletal muscle insulin action.

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